Multicenter study of ZAP-70 expression in patients with B-cell chronic lymphocytic leukemia using an optimized flow cytometry method

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Flow cytometry allows specific assessment of the expression of ZAP-70, a promising new prognostic factor in B-cell chronic lymphocytic leukemia (B-CLL), but suffers from a lack of multicenter standardization.

Design and Methods
An optimized method for direct detection of ZAP-70 in flow cytometry was tested in a multicenter fashion. Adapted for frozen cells, this method includes a normalization step by addition of B cells from a pool of peripheral blood mononuclear cells collected from normal donors. ZAP-70 expression levels were assessed for 153 patients with typical B-cell chronic lymphocytic leukemia chronic lymphocytic leukemia. Results were expressed as the ratio of ZAP-70 mean fluorescence intensity between B-CLL cells and normal B cells.

Results
The statistically optimized cut-off of ZAP-70 positivity was a ratio of 1.4. Concordance between ZAP-70 and CD38 expression was 67%. Concordance between the mutational status of *IgVH* genes and ZAP-70 or CD38 expression was 87% and 65%, respectively. ZAP-70 was significantly expressed in 28%, 54% and 61% of patients with Binet stages A, B and C B-cell chronic lymphocytic leukemia, respectively (p=0.008). The absence of ZAP-70 expression was associated with isolated del(13q14), a cytogenetic abnormality with a good prognosis, while most patients with the del(17p13) poor prognosis cytogenetic marker expressed ZAP-70 ($p<10^{-5}$). ZAP-70 expression was not related to the other poor prognosis cytogenetic abnormality del(11q22.3) nor to trisomy 12.

Conclusions
This new technique provides highly reliable results well correlated with the mutational status of *IgVH* genes, CD38 expression, Binet stage and cytogenetic abnormalities. This robust discriminative technique appears of particular interest for routine diagnosis and assessment of ZAP-70 expression in large, prospective, multicenter therapeutic trials.

Key words: B-cell chronic lymphocytic leukemia, ZAP-70, CD38, prognosis.

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Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a B-cell neoplasm characterized by an indolent course with progressive splenic and lymph node enlargement associated with chronic lymphocytosis. This disease is related to the accumulation of monoclonal B cells with the morphology of small mature lymphocytes. Typically, B-CLL cells exhibit a characteristic immunophenotype, co-expressing CD19, CD5 and CD23, in the absence or low expression of surface CD22, CD79b and FMC7. Surface immunoglobulins, usually IgM, -associated or not with IgD- are clonal but weakly expressed or undetectable. Patients with B-CLL follow heterogeneous clinical courses. Some survive for a long time without therapy, while others die rapidly despite aggressive treatment.[1] Until the end of the last century, only two major staging systems were used to address this clinical heterogeneity, both based on the number of enlarged lymph nodes associated or not with splenomegaly, anemia and/or thrombocytopenia.[2,3] These systems are still widely used and provide valuable prognostic information for identifying intermediate- and high -risk patients. However, neither of these staging systems allows an accurate prediction of the evolution of the disease among low risk patients or in the early stages of the disease.[4-7]

Recently, breakthroughs have been made in the identification of molecular and cellular markers that may predict disease progression. The first advance was made by Hamblin *et al.* and Damle *et al.*, who observed a correlation between the clinical course of B-CLL and the mutational status of immunoglobulin heavy chains variable region (*IgVH*) genes.[7,8] B-CLL cells with unmutated *IgVH* genes are associated with a poorer prognosis than those with mutated *IgVH*.[8,9] Additionally, cytogenetic abnormalities such as del(11q22.3), del(17p13), trisomy 12 and del 13q have been reported to be of significant prognostic value in B-CLL.[4,10] A correlation exists between unmutated *IgVH* genes and high-risk cytogenetic aberrations.[10,11] Although there is a general agreement that the mutational status of *IgVH* genes and cytogenetic abnormalities constitute strong and reliable prognostic factors for patients with B-CLL,[12,13] routine analysis, especially that of mutational status, is difficult, costly and inaccessible to most clinical diagnostic laboratories.

Among immunophenotypic markers also associated with prognosis in B-CLL, ZAP-70 expression is one of the most promising because of its strong correlation with *IgVH* mutational status.[14-17] ZAP-70, a tyrosine kinase initially described in the transduction complex of the T-cell receptor, is believed to increase the sensitivity of the B-cell receptor of CLL B cells to antigenic stimulation.[18] Compared to reverse transcriptase polymerase chain reaction or western blotting, flow cytometry allows a specific assessment of ZAP-70 levels in B-CLL cells,[15-17,19] avoiding the need to purify B CLL cells and to run the appropriate controls to check that purity.

However, ZAP-70 flow cytometric evaluation is currently a matter of debate because of the lack of standardization of technical procedures[20] for the proper detection of a versatile intracytoplasmic molecule located closely to the cell membrane's phospholipid bilayer. The three main concerns are the choice of the anti-ZAP-70 monoclonal antibody, the permeabilization procedure and data expression through normalization of the results and positivity threshold assessment.[7]

On behalf of the *Groupe d'Etude Immunologique des Leucémies* (GEIL), we evaluated various methods for the detection and quantification of ZAP-70 by flow cytometry in order to determine the possible relevance of this marker in future prospective multicenter therapeutic protocols. This study led us to develop and propose an optimized assay for determining ZAP-70 expression. This assay is improved in four points: (i) it allows the use of thawed cells; (ii) it includes an internal control consisting of peripheral blood mononuclear cells from a pool of normal donors; (iii) it uses a newly validated anti-ZAP-70 antibody[20] in a four-color combination; (iv) it incorporates an optimized fixation and permeabilization method. To validate this technique, we compared the detected levels of ZAP-70 expression with *IgVH* mutational status, CD38 expression, Binet stages and cytogenetic features in 153 B-CLL patients.

Design and Methods

Patients and collection of cells

For this study, 153 patients with B-CLL were enrolled according to institutional regulations and after approval from the scientific committees of the tumor banks of the University hospitals of Rouen, Bordeaux, Limoges and of Marseille's *Paoli Calmette* Cancer Institute. Patients were diagnosed or routinely followed between 2004 and 2006 in the four participating institutions (Bordeaux, Limoges, Marseille, Rouen) without curative treatment at the time of testing. Eleven additional cases and samples from normal subjects were also used for a multicenter reproducibility study. All B-CLL cases had a Matutes' score of 4 or 5.[21] Monotypic B cells were $CD5^+/CD23^+$ in all cases, and CD38 expression was assessed at diagnosis by flow cytometry. Blood samples (10 mL) from normal donors were obtained following institutionally approved protocols. Peripheral blood mononuclear cells were isolated from these normal blood samples, collected in EDTA-K, by Ficoll Hypaque gradient centrifugation, within 4 h after phlebotomy. Cells from each B-CLL patient, also separated on Ficoll Hypaque, were frozen in 10% dimethyl sulfoxide / 20% fetal calf serum at -80°C and stored in liquid nitrogen until the assay was performed. Cells from normal donors were either used freshly for optimization of ZAP-70 labeling or pooled and divided into aliquots before freezing in liquid nitrogen (normal pool).

ZAP-70 expression by flow cytometry

In a preliminary study, ZAP-70 labeling on cells from normal donors was performed either indirectly with the

unconjugated 2F3.2 clone (Upstate, United Kingdom), or directly with the Alexa 488–conjugated 1E7.2 clone (Caltag, Burlingame, CA, USA), the fluorescein isothiocyanate or phycoerythrin–conjugated SB70 clone (DakoCytomation, Trappes, France) or the phycoerythrin–conjugated SBZAP clone (Beckman Coulter, Miami, FL) according to previously described techniques for clones 2F3.2, 1E7.2 and SBZAP[16,19,21,22] and to the manufacturer's instructions for clone SB70.

Optimized ZAP-70 labeling of B-CLL cells was performed as follows. Upon rapid thawing and one wash in phosphate-buffered saline (PBS), the cells were incubated for 1 hour in RPMI medium (Eurobio, Courtaboeuf, France) at 37°C. After this period of rest, 10^5 cells from the normal pool were added to 5×10^5 cells of each B-CLL sample. These mixtures were then surface stained for 10 min at room temperature with fluorescein isothiocyanate-conjugated CD5 (BL1a), PECy5- (for use with an FC-500 flow cytometer), or APC- (for use with a FacsCanto flow cytometer) conjugated CD19 (J4.119) and PECy7-conjugated $CD3^+CD56$ (UCHT1+NKH1), all obtained from Immunotech (Marseille, France). The cells were then fixed for 10 min in 4% formaldehyde, and permeabilized for 30 min with Triton 10X (Sigma-Aldrich, Lyon, France) diluted 1:20 in PBS. After two washes with working buffer (PBS 0.1 mM, EDTA 2%, bovine serum albumin 0,1%, NaN_3, pH 7.2), the cells were incubated for 30 min with phycoerythrin-conjugated SBZAP (Beckman Coulter) diluted 1:10 in working buffer. After one wash, the cells were resuspended in PBS supplemented with 0.4% paraformaldehyde. Analyses were performed on either an FC500 (Beckman-Coulter Inc.) or a FacsCanto (Becton Dickinson, CA, USA) flow cytometer, using a gating strategy similar to that published by Shankey *et al.*[21] ZAP-70 expression was evaluated as the ratio of mean phycoerythrin fluorescence intensity between $CD19^+$ $CD5^+$ (B-CLL) and $CD19^+$ $CD5^-$ (normal control) B cells.

Analysis of IgVH gene sequences

After DNA extraction, *IgVH* genes were amplified by polymerase chain reaction (PCR) according to the BIOMED-2 protocol and purified PCR products were directly sequenced.[23] B-CLL *IgVH* sequences were aligned to germinal sequences on the IMGT database using V-QUEST and junction analysis software (http://imgt.cines.fr; initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) and the GenBank database using the IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/). *IgVH* genes with less than 98% sequence homology to the closest germ line counterpart were considered mutated as described elsewhere.[9,24]

Cytogenetics

Peripheral blood samples were cultured in the presence of tetradecanoyl phorbol acetate (TPA) as a mitogen and harvested after 72 hours. Relevant genetic aberrations were assessed by fluorescence *in situ* hybridization (FISH). A set of commercially available probes was used as follows: del(11q22.3), LSI ATM; an alpha satellite DNA probe CEP12 for trisomy 12; LSI D13S319 13q14 DNA probe; LSI D13S1020 13q34 DNA probe; LSI 17p13.1-P53 (Vysis, Downers Grove, IL, USA) and 6q21-D6S246 DNA probe (Qbiogen, Carlsbad, CA, USA). At least 200 interphase nuclei were evaluated per probe for each patient.

Statistical analysis

Means, standard deviations, χ^2 test, Student's t-test, sensitivities and specificities were calculated following standard statistical methods using the Statview software (Cary, NC, USA). Receiver operating characteristics (ROC) curves were established as described elsewhere.[25] To analyze any association between ZAP-70 expression and the lack of mutation of *IgVH* genes according to the ROC curve methodology, the percentage P of mutation of the *IgVH* gene was transformed as follows: tP = 2% - P, 2% representing the threshold for the identification of a mutated *IgVH* gene, so that the highest positive values of tP were for non-mutated *IgVH* cases, and the lowest values for mutated cases. The strength of the link between ZAP-70 expression and the absence of *IgVH* mutation was estimated by the area under the ROC curve.[25,26] ROC curves for CD38 and ZAP-70 expression were constructed using percentages and ratios, respectively.

Results

A preliminary study conducted on a small series of ten normal blood samples was performed by three of us (CA, FD, and AS) in order to evaluate the best method for ZAP labeling by flow cytometry, among those previously described in the literature. Whatever the technique, monoparametric analysis of ZAP-70 expression on normal peripheral blood mononuclear cells evidenced two peaks, corresponding to negative (B) and positive (T, NK) cells (Figure 1, left panel). The quality of discrimination between negative and positive cells was variable from one technique to the other. Our aim was to determine the method that best separated the peaks of positive and negative cells. With this criterion, it was found that the technique recommended by Shankey *et al.*[21], with the phycoerythrin-conjugated SZAP mononuclear antibody, paraformaldehyde fixation and Triton X100 permeabilization, yielded the best results with a mean fluorescence ratio of 11 between positive and negative cells (Figure 1, left panel), which was nearly double the next best ratio. This technique also appeared to better separate the peaks of ZAP-70 expression between T cells and CLL B-cells in patients expressing this kinase (Figure 1, right panel). These findings led us to select this method to further analyze the expression of ZAP-70 in B-CLL cells.

The gating strategy elaborated by Shankey *et al.*[21] is shown in Figure 2A on normal fresh cells. A preliminary study performed on normal subjects showed that ZAP-70

fluorescence levels of T and NK cells vary between individuals, whereas the barely detectable expression of ZAP-70 in normal B cells is remarkably stable, both in $CD5^-$ and $CD5^+$ B cells (Figure 2B and ref. #27). This suggested that using $CD19^+/CD5^-$ B cells as an internal control, as proposed by Shankey *et al.*[21], could result in more reproducible data. The addition of normal peripheral blood mononuclear cells to B-CLL samples prior to ZAP-70 labeling compensates for the lack of $CD19^+/CD5^-$ normal B cells in many B-CLL samples (Figure 3A). ZAP-70 results were then expressed as a ratio of the ZAP-70 mean fluorescence intensity between $CD19^+/CD5^+$(B-CLL cells) and $CD19^+/CD5^-$ (control B-cells) cells (Figure 3A). Notably, double analysis of a small series of B-CLL patients whose biological specimens were tested freshly and upon thawing confirmed unchanged B-CLL ZAP-70 ratios (Figure 3B). Therefore, for this study, a pool of normal peripheral blood mononuclear cells was prepared from peripheral blood samples of ten volunteer donors, forming a so-called *normal pool*. B-CLL cells from each patient and an aliquot of the normal pool were concomitantly thawed and mixed together as described above. To test whether our procedure was reproducible in a multicenter manner, 15 frozen samples (11 CLL cases and 4 normal samples) were distributed as frozen aliquots and tested in each of the four centers participating in this study. As shown in Figure 4, there was a good concordance between results from the centers, confirming the robustness of the procedure developed. Therefore, ZAP-70 levels were assessed in a series of 153 patients from the four centers. Information was collected on CD38 expression levels (151 patients), *IgVH* mutational status (135 patients), cytogenetics (141 patients) and Binet stage at diagnosis (125 patients).

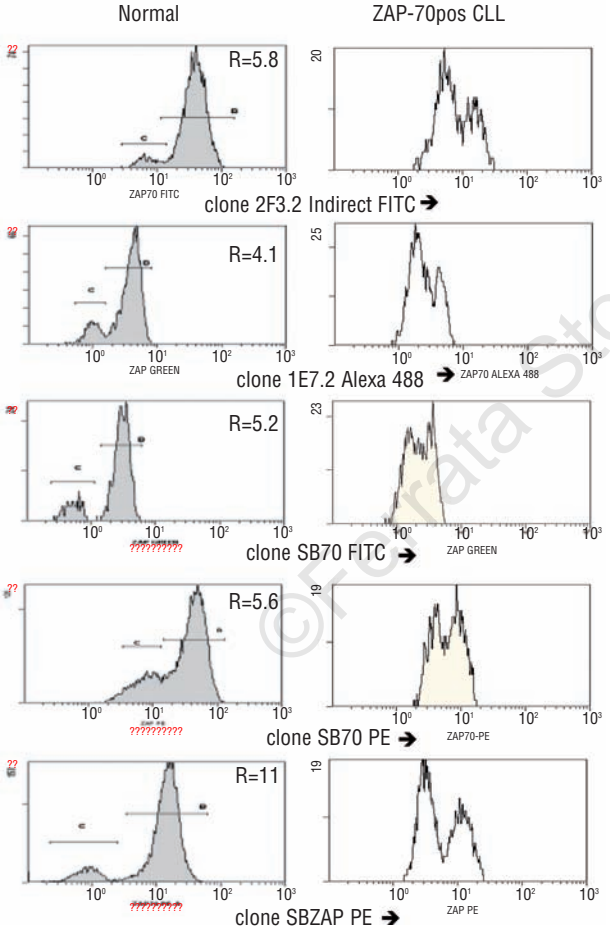

Figure 1. Comparison of monoparametric ZAP-70 labeling on peripheral blood mononuclear cells from normal donors (left panel) and from CLL patients (right panel) with different monoclonal antibody clones. Left panel: values of the ratio (R) between the mean fluorescence of the peaks of positive and negative cells are indicated within each histogram. Right panel: the number of B-CLL cells was adjusted to that of T cells in the gating so that, each time it was possible, both CLL B-cell and T-cell ZAP-70 peaks could be equilibrated and were well visible. The histograms presented here are from different subjects, chosen to be representative of normal donors and ZAP-70-positive B-CLL patients.

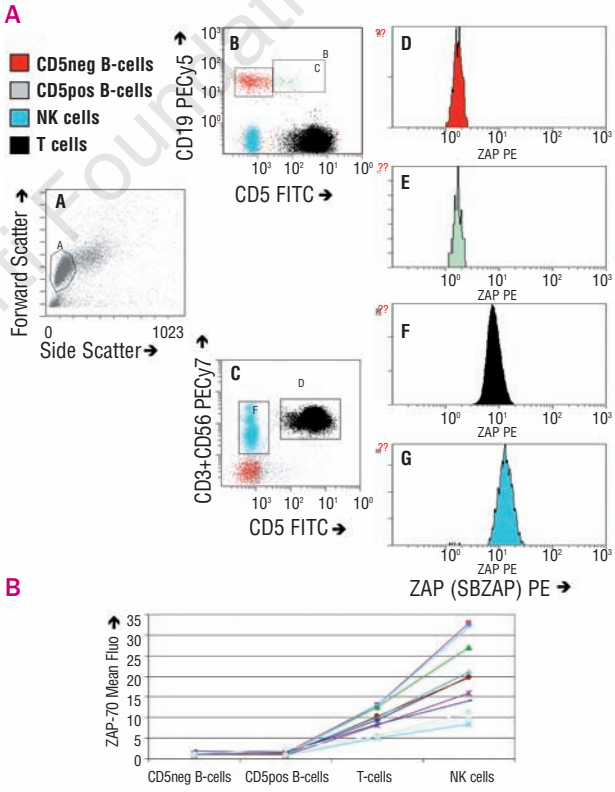

Figure 2. Flow cytometry analysis of CD5-FITC/ZAP-70-PE/CD3-PECy5/$CD3^+CD56^-$PECy7 staining on normal donors. Upon flow cytometry analysis, $CD5^-$ B cells were colored in red, $CD5^+$ B cells in gray, T cells in black and NK cells in cyan. (A) Gating strategy to assess ZAP-70 expression in the different cell compartments. Primary gating was performed on the SSC/FSC histogram (panel A) to eliminate debris. The CD5/CD19 histogram (panel B) allowed $CD19^+/CD5^-$ and $CD19^+/CD5^+$ B cells to be gated. Gating of T cells and NK cells was performed on the CD5/$CD3^+CD56$ histogram (panel C). ZAP-70 fluorescence was assessed on monoparametric histograms conditioned on each cell subset (panels D to G) as indicated by the color code. (B) Mean fluorescence intensity of ZAP-70 labeling in each cell subset from ten normal donors. For each cell subset, each dot represents one donor. Cells subsets from the same donor are joined by a line.

Patients with missing values are shown in Figure 5. Binet stage was A for 72%, B for 18% and C for 10% of the patients, of whom 70%, 37% and 33%, respectively, had mutated *IgVH* genes ($p<10^{-4}$). These proportions are similar to those reported by Damle *et al.*[8] As published by these authors, patients were classified according to the percentage of B-CLL cells expressing CD38 into two groups: one with 30% or more CD38$^+$ cells and the other with less than 30% CD38$^+$ cells. The concordance between CD38 and ZAP-70 expression was 67% ($p=5\times10^{-4}$), and that between CD38 expression and *IgVH* mutational status was 65% ($p=0.03$). Forty-two percent of CD38$^+$ cases and 63% of CD38$^-$ cases had *IgVH* mutated genes (χ^2 test, $p=0.03$). Plotting χ^2 values against the percentage of CD38$^+$ CLL B-cells did not evidence any maximum that could help to define an optimal threshold of CD38 expression when compared to the mutational status of *IgVH* genes (Figure 6A). ROC curve analysis clearly evidenced the weak link between CD38 expression and *IgVH* mutational status of CLL B cells (Figure 6B).

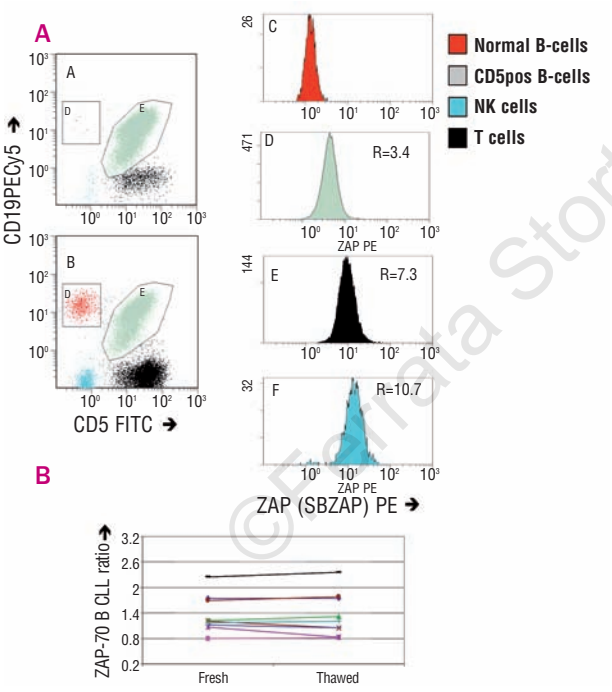

Figure 3. Determination of ZAP-70 ratios from thawed B-CLL samples (A) Addition of cells from a thawed sample of normal pooled PBMC to a B-CLL sample. Panel A: CD5/CD19 biparametric histogram for a typical thawed B-CLL sample. Panel B: CD5/CD19 biparametric histogram for the same B-CLL sample with addition of cells from a thawed sample of normal pooled PBMC. Note that both normal B cells and NK-cells were hardly detectable in the B-CLL sample without addition of cells from the normal pool. Panels C to F: ZAP-70 monoparametric histograms of CD5$^-$ normal B cells, CD5$^+$ B-CLL cells of a ZAP-70-positive patient, T and NK cells after addition of a thawed sample of normal pooled PBMC and following the gating strategy depicted in Figure 2. Fluorescence ratios (R) of B-CLL, T and NK cells to normal B cells are indicated in each corresponding histogram. (B) Comparison of ZAP-70 B-CLL cell/normal B-cell ratios (ZAP-70 B-CLL ratios) between fresh and thawed samples from the same patient. Each dot represents one patient. ZAP-70 B-CLL ratios from fresh and thawed samples of the same patient are linked.

Conversely, for ZAP-70, chi-squared maximization evidenced a unique peak corresponding to ratios between 1.3 and 1.4 (Figure 6A). This suggests that ZAP-70 ratios ranging between 1.3 and 1.4 would segregate patients equally well regarding the unmutated versus mutated status of B-CLL *IgVH* genes. Interestingly, only six patients (3.9%) had ZAP-70 ratios between 1.3 and 1.4, half of them with unmutated *IgVH* genes and the other half with mutated genes. This interval would thus correspond to a gray zone of ZAP-70 expression, being of undetermined significance regarding the mutational status of *IgVH* genes. ROC curve analysis of ZAP-70 ratios confirmed this interval, showing that it corresponds to the best compromise between specificity and sensitivity for prediction of the mutated or unmutated status of *IgVH* genes for B-CLL patients (Figure 6B). Sensitivity and specificity were, respectively, 87% and 86% for a threshold ratio of 1.3 and 81% and 91% for a threshold ratio of 1.4. ZAP-70 ratios were significantly lower in *IgVH* mutated than unmutated patients (Figure 7A, Student's t test, $p<10^{-6}$). The concordance between ZAP-70 expression and *IgVH* gene mutational status was 87% (Figure 7B).

With the decisional threshold of 1.4 for ZAP-70 ratios, we observed that 28%, 54% and 61% of patients with stages A, B and C B-CLL, respectively, expressed enhanced levels of ZAP-70 (Table 1, *online supplement*, χ^2: $p=0.008$), confirming a significant association between ZAP-70 expression and Binet stage C on the one hand, and Binet stage A and absence of ZAP-70 expression on the other hand, with a concordance of 70%, close to previously published data.[28,29]

ZAP-70 expression also correlated with cytogenetics (Table 2, *online supplement*). Indeed, most patients with the good prognosis isolated del(13q14) cytogenetic abnormality were negative for ZAP-70 while most patients with the del(17p13) poor prognosis cytogenetic marker expressed ZAP-70 (Table 2, *online supplement*, $p=2\times10^{-5}$). Interestingly, we found no significant association between ZAP-70 expression and either trisomy 12 or the del(11q22.3) cytogenetic marker, another poor prognosis abnormality. ZAP-

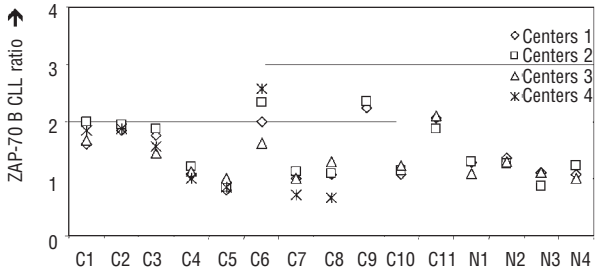

Figure 4. Multicenter comparison of ZAP-70 B-CLL ratios. Eleven CLL (C1 to C11) and four normal (N1 to N4) frozen samples of 5×10^6 cells were distributed among four different centers. ZAP-70 B-CLL ratios were determined using thawed aliquots of the same normal pooled peripheral blood mononuclear cells sample as the internal control. Values of ZAP-70 B-CLL ratios are plotted for each center.

70 expression levels were comparable in ZAP-70 positive cases with or without poor prognosis cytogenetic abnormalities. As for ZAP-70 expression, we observed that most patients with isolated del(13q14) had mutated *IgVH* genes while most patients with del(17p13) had unmutated genes (Table 2, *online supplement*, $p<10^{-6}$). Among the six cases with ZAP-70 ratios between 1.3 and 1.4, cytogenetic data were not available in one case, isolated del13q was found in three cases and del13q plus trisomy 12 in the last. We did not find any significant association between *IgVH* mutational status and trisomy 12, yet we observed such an association with del(11q22.3), most of the patients harboring this deletion having unmutated *IgVH* genes (Table 2, *online supplement*). These data suggest that ZAP-70 expression is related with some, but not all, poor prognosis cytogenetic abnormalities. Moreover, it seems likely that, even if strongly related, the biology of ZAP-70 dysregulation is not directly linked to the mutational status of *IgVH* genes since the association of these two parameters with cytogenetic abnormalities is different.

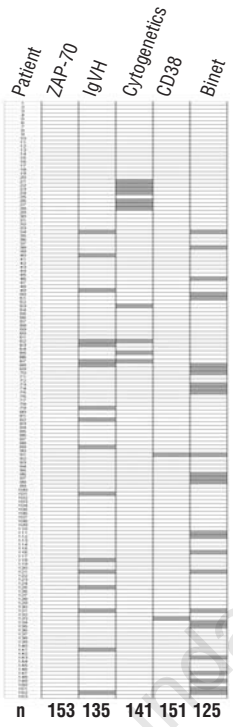

Figure 5. Collected information for the comparison of ZAP-70 B-CLL ratios with other CLL prognostic markers. The collected information is indicated at the top. For each piece of information collected, the number of patients is indicated at the bottom. Gray rectangles represent missing information.

Discussion

In this study, we report on the strong correlation between ZAP-70 expression, Binet stage, *IgVH* mutational status and cytogenetics in 153 patients with B-CLL, using a new optimized flow cytometry protocol of ZAP-70 detection in CLL B cells. Our results confirm that ZAP-70 expression is closely associated with the unmutated status of *IgVH* genes in B-CLL. Furthermore, our results clearly show that ZAP-70 expression is associated with some but not all cytogenetic markers of poor prognosis, whereas lack of ZAP-70 expression is related to cytogenetic markers of good prognosis.

Various prognostic markers for the evolution of B-CLL have been reported in the literature, such as soluble CD23, thymidine kinase levels, and expression of CD38 or ZAP-70.[12,13,30] Our results clearly demonstrate that ZAP-70 is a much better predictor of the mutational status of *IgVH* genes in B-CLL than is CD38. ZAP-70 expression was initially thought to be restricted to T cells, but upregulation of this kinase has recently been reported in normal B-cell subsets as well as in various B-cell neoplasms.[31] Transcriptome studies first showed that B-CLL cells upregulate ZAP-70 expression; it was then shown that this upregulation was restricted to patients with unmutated *IgVH* genes.[14] This initial relationship between increased expression of ZAP-70 and absence of *IgVH* gene mutations in B-CLL was repeatedly confirmed,[13-15,17,28,32] suggesting that the determination of ZAP-70 expression could be a surrogate marker, easier to investigate than mutational status. To date, ZAP-70 is indeed considered the most reliable prognostic immunophenotypic marker for B-CLL.[34]

When considering both the expression of ZAP-70 and *IgVH* mutational status, three groups of B-CLL patients

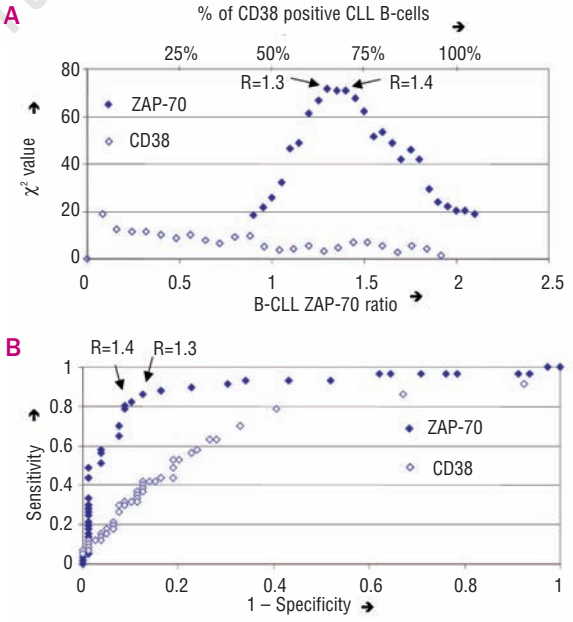

Figure 6. Determination of the optimal threshold of ZAP-70 and CD38 levels predicting the mutational status of IgVH genes in B-CLL. (A) χ^2 values for the distribution of B-CLL patients in the four categories defined by mutated or unmutated status of *IgVH* genes versus positivity or negativity of ZAP-70 or CD38 expression. The lower horizontal scale corresponds to ZAP-70 ratios, and the upper horizontal scale to the percentage of CD38 positive cells. (B) ROC curve analysis between ZAP-70 or CD38 expression and mutated versus unmutated status of *IgVH* genes. To calculate the successive χ^2 values (Figure 3A) and the successive specificities and sensitivities (Figure 3B), the mutation threshold level was fixed at 2% and thresholds ranged between 0.5 and 6 with a step of 0.05 for ZAP-70 ratios and between 0% and 100% with a step of 4% for CD38 positive cells.

can be distinguished: those with a mutated *IgVH* gene and absence of ZAP-70 expression, those with unmutated *IgVH* genes and ZAP-70 expression and those with a discordance between ZAP-70 expression and the mutational status of IgVH genes. Most authors accept that the discordant cases belong to a gray zone of undetermined significance for ZAP-70 expression, corresponding to intermediate ZAP-70 expression levels. The current methodological problem is that the breadth of this gray zone is dependent both on the technique of ZAP-70 detection and on the method used to establish the level of ZAP-70 expression.[7,35] As regards the technique, various clones of monoclonal antibodies, methods of cell permeabilization and calculation of ZAP-70 expression have been used. A recent review of the literature, comparing these variables, concluded that it was not currently possible to reach any consensus.[20] This conclusion was emphasized at the time of publication by the knowledge that new reagents would likely soon be available.[20]

The comparative study of different clones and techniques, performed within the GEIL, led us to select the new phycoerythrin-conjugated SBZAP monoclonal antibody, shown to be the most discriminative between negative and positive cells. As mentioned above, one of the main problems was to define the correct control for ZAP-70 expression. Moreover, the accuracy and reproducibility of the results may depend on the calculation method. Both Rassenti *et al.*[17] and Crespo *et al.*[15] proposed expressing the results as the percentage of positive cells above a given fluorescence threshold channel, defined by normal B cells from a healthy donor or the residual T-cell population, respectively. Letestu *et al.*[20] showed that this threshold-based calculation method is poorly reproducible in multicenter studies and proposed expressing the results as the mean fluorescence ratio between normal residual T cells and B-CLL.[20] Both these calculation methods are based on the wrong assumption that T-cell expression levels of ZAP-70 are homogeneous from one individual to the other. Shankey *et al.*[34] alternately proposed expressing ZAP-70 results as a ratio between the mean fluorescence intensity of B-CLL and that of residual normal B cells. We confirmed the poor multicenter reproducibility of the calculation methods proposed by Crespo *et al.*[15] and Rassenti *et al.*[17] (*data not shown*). When analyzing our data, we observed that the ZAP-70 ratios obtained in our sample series by plotting B-CLL values versus those of normal B cells were linear while the ratios calculated using the fluorescence levels of normal T cells versus B-CLL cells were not linear (*not shown*). In fact, when compared to the *IgVH* mutational status, ZAP-70 T-cell/B-CLL ratios were as specific as ZAP-70 B-CLL/normal B-cell ratios but less sensitive (*not shown*). Thus, in our experience, normal B cells appear to be the best control, because of the very low and highly stable level of ZAP-70 expression (or non-expression) in these cells. Conversely, the constitutive expression of ZAP-70 in T and NK cells engaged in variable levels of immunological activity in different individuals leads to highly heterogeneous results in these lymphocyte subsets (as shown by our results and Zucchetto *et al.*[27]). Moreover, assessing ZAP-70 on frozen/thawed B-CLL samples further enhances, with a minimal extra cost, the standardization of the technique because it allows the results from a series of patients to be normalized to the same pool of normal peripheral blood mononuclear cells. Finally, the robustness of the method was validated by the concomitant testing of 11 B-CLL and four normal subjects in the four centers. This experiment yielded nearly identical results and, notably, no modification of the ZAP-70 positive or negative status of the B-CLL samples.

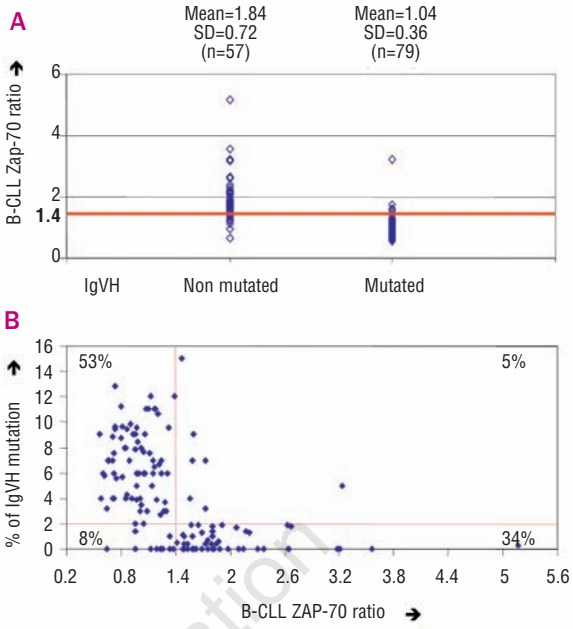

Figure 7. Analysis of B-CLL ZAP-70 ratio in patients with non mutated and mutated *IgVH* genes. (A) ZAP-70 expression levels in B-CLL patients. Mean and standard deviation (SD) of ZAP-70 ratios in patients with non mutated or mutated *IgVH* gene are indicated above each graph. The 1.4 decisional threshold of ZAP-70 ratio positivity is indicated by the red line. Patients numbers in each category are indicated above the graph in brackets. (B) Biparametric graph of concordance between ZAP-70 ratios and percentages of *IgVH* genes mutation in B-CLL patients. The vertical bar corresponds to the 1.4 ZAP-70 ratio threshold, and the horizontal bar to the 2% threshold for *IgVH* gene mutations. Percentages of each category are indicated within the graph.

With this technique, we observed a stronger concordance between ZAP-70 expression and *IgVH* gene mutational status than previously published.[10,17,33] Using the χ^2 maximization procedure allowed the statistical and objective definition of a small gray zone of ZAP-70 expression for ratios between 1.3 and 1.4. Only 3.9% of the patients in our series fell in this interval, which corresponds to the range of non-decisional ZAP-70 ratios for prediction of the mutational status of IgVH genes. We also confirmed the association between ZAP-70 expression and Binet stages[28] and corroborated and extended the cytogenetic study of Kröber *et al.*[29] Comparisons of both ZAP-70 expression and *IgVH* mutational status with cytogenetic

data showed that both ZAP-70 expression and unmutated *IgVH* genes were associated with the poor prognosis del17p cytogenetic abnormality whereas the good prognosis del13q abnormality was associated with mutated *IgVH* genes and lack of ZAP-70 expression. Additionally, the del11q abnormality was associated with unmutated *IgVH* genes in CLL patients but not with ZAP-70 expression. This is a clear indication that selection of unmutated transformed CLL B cells and ZAP-70 expression are not directly related and raises the question of the mechanism of ZAP-70 dysregulation.

The definition of the best technical conditions for ZAP-70 detection and quantification is very important to in order to usefully compare this phenotypic marker against *IgVH* mutational status and cytogenetic markers in future treatment protocols that may stratify therapeutic approaches according to these parameters. It is also essential for reliable, routine staging of patients for whom the technique reported here would be of particular interest. Furthermore, the fact that cells can be stored and used in batch analysis would greatly facilitate the evaluation of ZAP-70 in large, prospective, multicenter therapeutic protocols. The importance of ZAP-70 expression in stratifying patients for different therapeutic options and from an economic point of view will then have to be determined.

Authorship and Disclosures

NG: contributed to setting up the new method, performed analysis of mutational status of *IgVH* genes and cytogenetics on samples from Limoges, analyzed data, contributed to writing the manuscript; AS contributed to setting the new method in Limoges, produced and analyzed flow cytometry data; CB: performed the new method in Rouen, produced and analyzed flow cytometry data; CA performed the new method in Marseille, produced and analyzed flow cytometry data; FD participated in the comparisons of the preliminary study; BL performed the new method in Rouen, produced and analyzed flow cytometry data; SL provided patients' samples from Rouen; SO performed analysis of mutational status of *IgVH* genes on samples from Marseille; FJ provided patients' samples from Rouen; ML-P performed cytogenetics studies on patients from Marseille; DP performed analysis of mutational status of IgVH genes and cytogenetics on samples from Rouen; DS performed the new method in Marseille; LR provided patients' samples in Limoges; JF initiated and constructed the study, wrote the manuscript; MCB discussed the study, wrote and edited the manuscript. The authors reported no potential conflicts of interest.

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